Parenteral Vaccination Can Be an Effective Means of Inducing Protective Mucosal Responses

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The current paradigm in vaccine development is that nonreplicating vaccines delivered parenterally fail to induce immune responses in mucosal tissues. However, both clinical and experimental data have challenged this concept, and numerous studies have shown that induction of mucosal immune responses after parenteral vaccination is not a rare occurrence and might, in fact, significantly contribute to the protection against mucosal infections afforded by parenteral vaccines. While the mechanisms underlying this phenomenon are not well understood, the realization that parenteral vaccination can be an effective means of inducing protective mucosal responses is paradigm-shifting and has potential to transform the way vaccines are designed and delivered.

Despite the availability of vaccines and therapeutics, infectious diseases are the world's leading killers of children and young adults. They account for more than 10 million deaths a year—1 in 2 deaths in developing countries. Most deaths from infectious diseases—almost 90%—are caused by only a few diseases (pneumonia, tuberculosis, diarrheal diseases, malaria, and human immunodeficiency virus [HIV]/AIDS). Unfortunately, traditional vaccine strategies have failed to provide effective protection against the majority of these diseases. For most of these infections, the first contact between the disease-causing microorganism and the human host occurs at mucosal surfaces, specifically, at the nasal, oropharyngeal, respiratory, gastrointestinal, and urogenital mucosa. Ideally, a vaccine would induce not only systemic but also mucosal immune responses, including production of effector lymphocytes and antibodies capable of interfering with microbial adhesion, neutralizing bacterial toxins, or even inactivating pathogens inside epithelial cells.

The current paradigm in vaccine development is that nonreplicating vaccines delivered parenterally (i.e., by needle injection under the skin) fail to induce immune responses in mucosal tissues. However, both clinical and experimental data have challenged this concept, and numerous studies have shown that induction of mucosal immune responses after systemic vaccination is not a rare occurrence and might, in fact, significantly contribute to the protection against mucosal infections afforded by parenteral vaccines. Furthermore, recent studies indicate that formulating parenteral vaccines with mucosal trafficking-targeted components induces homing receptor expression on T cells and B cells and their subsequent migration to mucosal compartments. The realization that parenteral vaccination can be an effective means of inducing protective mucosal responses is paradigm-shifting and has unparalleled potential to transform the way vaccines are designed and delivered.

Many instances of parenteral vaccine-induced mucosal responses have been reported in humans, nonhuman primates, and other experimental animals. A few illustrative examples in humans include the following: significant increases in levels of antigen-specific IgA and/or IgA antibody-secreting cells (ASCs) in saliva, tonsils, or vaginal or oral fluids after systemic immunization with tetanus toxoid (TT), inactivated or subvirion influenza vaccines, meningococcal and pneumococcal polysaccharides, and *Haemophilus influenzae* capsular polysaccharide. As early as 1973, Ogra and Ogra (1) detected the presence of antipoliovirus (anti-PV) IgG in vaginal washes of women immunized intramuscularly with inactivated poliovirus vaccine (IPV). The presence of salivary anti-influenza IgG, IgM (2), and IgA (3) was demonstrated following intramuscular immunization with the trivalent split influenza vaccine. Interestingly, subcutaneous vaccination of human volunteers with a trivalent split influenza vaccine induced significant increases in levels of IgA and IgM but not IgG ASCs in the tonsils. The authors speculated that IgA-committed activated B cells homed to mucosal tissues from the draining lymph nodes of the vaccination site. More recently, Halperin et al. (4) observed that intramuscular vaccination of postpartum women with tetanus-diphtheria-acellular pertussis vaccine (Tdap) induced secretion of IgA against pertussis antigens in breast milk. One of the most interesting demonstrations of mucosal antibodies following nonmucosal immunization was the detection of urine and stool anti-toxin IgA following transcutaneous immunization with the heat-labile enterotoxin (LT) of *Escherichia coli* (5).

Data from nonhuman-primate studies add to the complexity of the available information regarding mucosal responses induced by parenteral immunization (6–10). Cheng and colleagues observed that rhesus macaques vaccinated with an outer membrane protein from *Chlamydia trachomatis* developed antigen-specific IgA and IgG in stools, saliva, tears, and vaginal washes. Others have reported the development of mucosal immunity and protection against oral challenge with wild-type virus after parenteral vaccination of monkeys with IPV and increases in levels of salivary IgA and protection against challenge with a parenterally administered *Shigella* ribosomal vaccine. Numerous studies in nonhuman primates vaccinated parenterally with HIV- or simian immuno-

deficiency virus (SIV)-derived nonreplicating antigens have demonstrated that antigen-specific humoral and cellular mucosal immune responses can be elicited equivalent to that obtained after administration of the vaccines directly onto mucosal surfaces. In spite of the moderate responses noted in these studies, tetramer-positive CD8$^+$ T cells were observed in the gut and high-avidity mucosal cytotoxic T lymphocytes (CTLs) were present in regional lymph nodes after subcutaneous vaccination of macaques with HIV/SIV peptides.

Studies using murine or other small-laboratory-animal models have also provided relevant, although at times inconclusive, evidence of mucosally committed responses after parenteral delivery of vaccines (11–15). A few important examples include reports of long-lasting mucosal IgA responses in intestinal tissues of BALB/c mice after intramuscular injection of nonadjuvanted tetanus toxoid (TT) and persistent levels of intestinal and vaginal antibodies after intramuscular immunization of mice with an HIV gp41 peptide mixed with a major histocompatibility complex class II (MHC-II) binding peptide. Enioutina and colleagues described the generation of neutralizing IgA and IgG antibodies against *Haemophilus influenzae* in nasal and vaginal secretions after subcutaneous injections of capsular saccharides conjugated to diphtheria toxoid and mixed with vitamin D3. The same group of investigators demonstrated that subcutaneously administered Toll-like receptor (TLR) ligands (TLR3 and -4) that induce increases in levels of 1α-hydroxylase (the enzyme responsible for generation of the active form of vitamin D3) in dendritic cells (DCs) alter DC migratory patterns *in vivo* and promote the induction of mucosal immune responses to coadministered antigens, apparently as a consequence of antigen-bearing DCs migrating to both draining and nondraining lymph nodes, especially the Peyer's patches. A subsequent study confirmed the role played by retinoic acid (RA) in mucosal responses after subcutaneous vaccination of mice with ovalbumin (OVA) and RA. In that study, T cells from RA-treated mice expressed the mucosal homing integrin α4β7 and migrated from the skin-draining lymph nodes to the gut (13–15). The majority of the studies described above were limited to the quantification of humoral (IgA and/or IgG) responses in the mucosal compartment, with little or no attention given to the cellular and/or molecular inductive mechanisms responsible for the observed outcomes.

Two leading hypotheses have been advanced to explain this phenomenon: antigen is captured locally by antigen-presenting cells (APCs) and carried to mucosal sites for antigen presentation, or, conversely, antigen is presented peripherally to naive T cells and B cells which are then imprinted to home to mucosal tissues. These are not mutually exclusive processes. However, conventional immunology dictates that mucosal immune responses can best be generated by presenting antigen to cells in the mucosa-associated lymphoid tissues (MALT) (the current paradigm). To suggest otherwise would require an entire reexamination (and rethinking) of what is known about mucosal immunity and communication between the systemic and mucosal immune compartments.

Brief description of the current paradigm for inducing mucosal immunity. According to the current paradigm, mucosal immunity is initiated when a foreign antigen is encountered by an APC in the mucosal compartment. As shown in Fig. 1, mucosal surfaces maintain a constant vigil against pathogens, mediated in part by continuous antigen sampling by APCs, including dendritic

cells (DCs), macrophages, and B cells. APCs are important initiators of adaptive immune responses and vaccine-induced immunity and often have discrete functions and subsets. For example, a recent study demonstrated that CX3CR1$^+$ DCs promote Th1/Th17 cell differentiation, whereas CD103$^+$ DCs induce T regulatory cell differentiation in mouse colonic lamina propria (16). Another specialized cell type, the microfold (M) cell, transports substances across the epithelial surface for subsequent uptake and processing by DCs for initiation of immune responses (17). These DCs prime naive T cells to clonally expand and differentiate into effector T cells, including helper T-cell subsets (Th1, Th2, Th17, or T regulatory cells), cytotoxic T lymphocytes (CTLs), and memory T cells. Tissue-specific expression of chemokine and chemokine receptors on memory/effector cells contributes to their preferential mucosal localization and retention. For example, IgA plasmablasts expressing CCR9 bind to the CCL25 ligand produced in the small intestine, whereas IgA plasmablasts expressing CCR10 bind to the CCL28 ligand found in the large intestine. Gut-associated lymphoid tissue (GALT)-derived DCs are able to imprint T cells and plasma cells with specific mucosal homing molecules. In contrast, peripheral lymph node DCs confer expression of CXCR3, allowing responsiveness to CXCL9 and CXCL10 and migration to inflamed mucosal and nonmucosal tissues.

Role of adjuvants in imprinting of naive T and B cells. An important aspect of this model is that imprinting of naive T and B cells takes place in the mucosal compartment. However, recent evidence from our laboratory and the laboratories of others has shown that a small number of adjuvants (some of the TLR agonists and bacterial ADP-ribosylating toxin adjuvants) can promote mucosal imprinting following parenteral immunization; most cannot. Our own efforts over the last 3 decades have focused on use of the heat-labile enterotoxin (LT) of enterotoxigenic *E. coli* (ETEC) as an adjuvant that induces mucosal and systemic immune responses regardless of the route of administration (Fig. 2).

LT is closely related to cholera enterotoxin (CT) produced by *Vibrio cholerae*, and the adjuvant properties of these molecules have been known for some time. A number of investigators have introduced genetic mutations into LT in attempts to detoxify the molecule and make it safe for inclusion as an antigen (i.e., in an ETEC vaccine) or for use as an adjuvant. Most of these efforts have focused on the sites within LT where NAD binds and is hydrolyzed. Creation of the adjuvant double mutant LT (dmLT), or, more technically, LT(R192G/L211A), involved introduction of purposeful stepwise mutations into the LT holotoxin A subunit, based on how the holotoxin interacts with mammalian cells. The combined R192G/L211A mutations in dmLT prevent proteolytic activation of the molecule, reduce enzymatic activity by >1,000-fold, completely eliminate enterotoxicity, and preserve the full adjuvant properties of native LT (18). dmLT has been shown in numerous preclinical and clinical studies to elicit both humoral immunity and cellular immunity to coadministered antigens from a variety of bacterial and viral pathogens in both the systemic and mucosal compartments following either mucosal or parenteral delivery (19).

In a series of recently completed studies (20), we examined the impact of combining dmLT with trivalent IPV for dose sparing, inducing mucosal immunity, and increasing the longevity of antipoliovirus (anti-PV) responses in a mouse model following either intradermal or intramuscular delivery. We found that non-
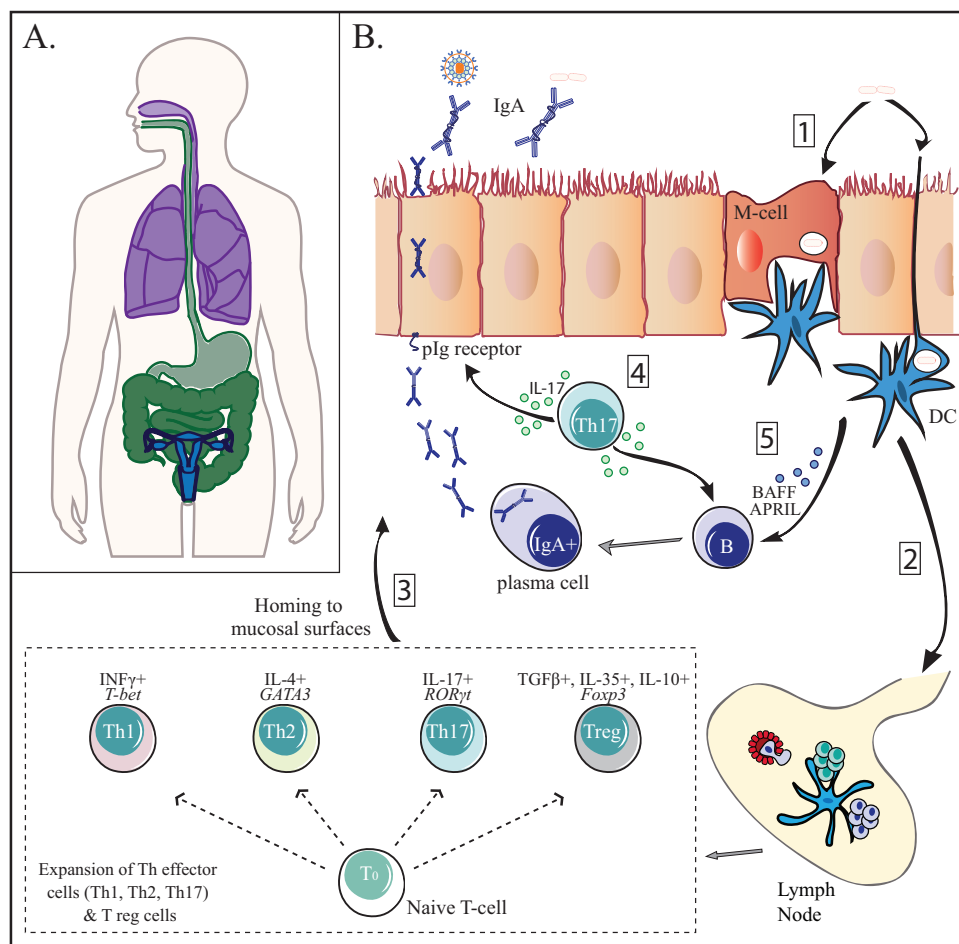
FIG 1 Mucosal immunity. (A) Mucosal surfaces constitute the largest interface between the body and the external environment, including the respiratory (purple), gastrointestinal (green), and genital (blue) tracts. (B) Mucosal immunity plays a crucial role in defense against invading pathogens at the epithelial cell surface, involving a complex network of innate and adaptive immune components. Continuous pathogen surveillance is mediated by specialized antigen transport cells (M cells) and antigen processing cells (DCs) (step 1). Mucosal DCs are particularly important for initiating adaptive immune responses by migrating to the draining lymph node and mediating the expansion of antigen-specific naive T cells into T helper subsets (step 2), involving an upregulation of transcription factors (T-bet, GATA3, RORgt, or Foxp3) and lineage-defining cytokines (gamma interferon [IFN-γ], interleukin-4 [IL-4], IL-17, transforming growth factor β [TGF-β], IL-35, and IL-10). Expanded T-cell subsets home back to mucosal surfaces to perform their effector functions (step 3). Th17 cells and IL-17 expression can upregulate polymeric Ig (pIg) receptor expression and IgA class switching, enhancing IgA secretion (step 4). In addition, soluble factors (BAFF, APRIL) secreted by DCs and epithelial cells can promote T-cell-independent IgA class switching (step 5). Increased IgA production and translocation through epithelial cells hinder pathogen invasion and promote immunity at mucosal surfaces. (Republished from reference 21 with permission of the publisher.)
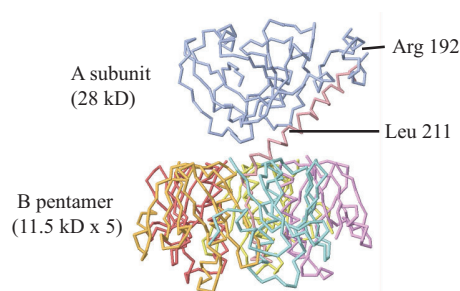

FIG 2 Structure of LT. The amino acid backbone diagram shows partially active LT, with identification of subunits and locations of the two mutations (R192G and L211A) present in dmLT. (Republished from reference 18.)

adjuvanted intradermal delivery was not superior to intramuscular delivery for fractional dose sparing but was associated with development of mucosal immunity. Vaccination with IPV plus dmLT promoted serum anti-PV neutralizing antibodies with fractional IPV doses by either intramuscular or intradermal delivery, achieving at least 5-fold dose sparing above the levels seen with nonadjuvanted fractional doses. dmLT also promoted germinal center formation and longevity of serum anti-PV neutralizing titers. Importantly, dmLT enhanced mucosal immunity, as defined by fecal and intestinal anti-PV IgA secretion, when included in IPV immunization by intradermal or intramuscular delivery.

Our collaborators have also generated data indicating that inclusion of dmLT in a parenteral vaccine formulation influences the imprinting of homing mucosal receptors on trafficking T cells. Using novel tetramer technology, D. R. Frederick and J. B. McLachlan, Tulane University School of Medicine (personal

communication), tracked antigen-specific CD4$^+$ T cells after intradermal vaccination in the presence of dmLT. This immunization results in T-cell upregulation of the $\alpha 4\beta 7$ gut mucosal homing marker in draining lymph nodes and mucosal lymph nodes and the presence of antigen-specific CD4$^+$ T cells trafficking to the colon. Inclusion of dmLT in the vaccine also led to increased frequencies of dermal CD103$^+$ dendritic cells and Langerhans cells. As reported previously, dmLT imparted a balanced Th1/Th2/Th17 phenotype.

We believe that these findings are a significant step in understanding how antigen-specific CD4$^+$ T cells can be manipulated by adjuvant/antigen formulations to alter phenotype and tissue destination. Further, this work has provided new insights into linking innate responses by antigen-presenting cells and downstream antigen-specific T-cell responses.

Conclusion and perspectives. The concept of specifically targeting mucosal surfaces (effector phase) by manipulating the inductive phase of parenteral vaccination has been addressed only recently. Consequently, many unresolved questions remain: specifically, how are these responses generated, what are the molecular events that determine migration to mucosal tissues, what inductive cellular mechanisms support development of protective responses in mucosal tissues, and what are the qualities of the T- and B-memory cells generated in the mucosa after systemic immunization? Induction of mucosal immune responses following parenteral immunization is paradigm-shifting and has the potential to transform the way vaccines are designed and delivered.

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